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ORIGINAL ARTICLE

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Acidic dopamine metabolites are actively extruded from PC12 cells by a novel sulfonylurea-sensitive transporter

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Abstract Incubation of PC12 cells with the sulfonylurea drug, glipizide (1–100 μM), increased intracellular levels of the acidic metabolites of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The levels of these acids in the medium were decreased, indicating the presence of a sulfonylurea-sensitive organic anion transporter. In the present study, we demonstrate that the sulfonylurea-sensitive transport of acidic dopamine metabolites is unidirectional, ATP dependent, unaffected by ouabain or by tetrodotoxin and blocked by drugs that interact with the multidrug-resistance protein-1 (MRP1). However, over-expression of MRP1 did not affect transport of the acid metabolites. The pharmacological profile and ion dependence of the transporter also differs from that of known ATP-binding cassette (ABC) family members. Using microdialysis, we also demonstrated a sulfonylurea-sensitive transport process in the striatum of freely moving rats. These results show that acidic dopamine metabolites are actively secreted from dopaminergic cells into surrounding extracellular fluid by a previously undescribed transporter.

Key words 3,4-Dihydroxyphenylacetic acid · Homovanillic acid · Dopamine · Transporter · Sulfonylurea · PC12 cells · ATP-binding cassette (ABC)

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Introduction

3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are deaminated acidic dopamine metabolites (ADMs) that are actively transported from the cerebrospinal fluid into plasma by an energy-dependent, probenecid-sensitive transporter (Gordon et al. 1976). It has generally been assumed that DOPAC and HVA, once formed in brain cells, simply diffuse into the extracellular space and that under conditions in which the efflux of these metabolites from extracellular fluid is unaltered, their steady state levels reflect the production rate of dopamine (Westernik and Kikert 1986; Arbuthnott et al. 1990; Cumming et al. 1992). There is some evidence, however, that dopaminergic neurons actively transport DOPAC into the extracellular fluid. Miyamoto et al. (1993) attributed the potassium-induced decrease in extracellular levels of DOPAC and HVA to a blockade of export of the acidic metabolites from dopaminergic nerves into the extracellular fluid. Also, under conditions of ischemia, extracellular levels of DOPAC and HVA are decreased, whereas tissue levels of these metabolites are increased (Phebus et al. 1995), suggesting that there is an energy-dependent active transport of the metabolites out of brain cells.

We recently found that treatment of cultured PC12 cells with glipizide (1–100 μM), a sulfonylurea that blocks ATP-sensitive potassium (K_{ATP}) channels, increases intracellular concentrations of the ADMs, DOPAC and HVA, while decreasing their entry into the culture medium. This suggested the presence in PC12 cells of an organic anion transporter for ADMs that is blocked by sulfonylureas (Lamensdorf et al. 2000).

In the present study we functionally characterize the ADM transporter that is blocked by glipizide. First, we examined the components of K_{ATP} channels as possible mediators of ADM transport. K_{ATP} channels are octomers composed of four molecules each of two distinct proteins. One protein (Kir6.1 or Kir6.2) forms the pore of an inwardly-rectifying K-channel, whereas the second regulates the channel activity and is one of three subtypes of a sulfonylurea receptor (SUR1, SUR2A or SUR2B) protein (Inagaki et al. 1995, 1996; Sakura et al. 1995; Clement et al. 1997; Aguilar-Bryan et al. 1995). Although none of the SUR proteins have been shown to function as transporters, structurally, they belong to the ATP-binding cassette (ABC) transporter family (see Tusnády et al. 1997). We also compared the effects on DOPAC extrusion from PC12 cells of a variety of drugs known to affect ABC transporters. In addition, using cells over-expressing the multidrug-resistance protein-1 (MRP1, a known organic anion transporter), we tested the possibility that this transporter could be responsible for the transport of ADMs. We also tested the in vivo relevance of our finding using microdialysis to examine the effect of a sulfonylurea and an MRP blocker on striatal extracellular DOPAC and HVA levels.

Materials and Methods

Cell culture and solutions

Stock PC12 cells were obtained from the American Type Culture Collection (Manassas, Va., USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g of glucose/l, 10% heat-inactivated horse serum, 5% fetal calf serum (FBS), 100 units/ml penicillin and 0.05 mg/ml streptomycin in 5% CO_2 atmosphere at 37 °C. Medium was changed every 3 days and the cells were subcultured about once a week. When PC12 cells were to be differentiated, 100 nM nerve growth factor (NGF) was added to the cultured medium and incubation continued for 6 days.

NIH3T3 cells that had been transfected with vector alone or with the *MRP1* gene (Breuninger et al. 1995) (originally prepared by Dr. Gary Kruh and obtained as a gift from Dr. Suresh Ambudkar, DBS, NCI) were maintained in DMEM (4.5 g glucose/l) supplemented with 10% FBS, 100 units/ml penicillin and 0.05 mg/ml streptomycin containing 0.75 mg/ml G418 in 5% CO_2 atmosphere at 37 °C.

NIH3T3 and NIH3T3-MDR1-G185 cell lines were grown as monolayer cultures at 37 °C in 5% CO_2 in DMEM supplemented with 4.5 g/l glucose, 5 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FBS. The drug-sensitive NIH3T3 cells were grown in the absence of colchicine. NIH3T3-MDR1-G185 cells are NIH3T3 cells transfected with the wild-type (G185) pHa-MDR1/A retroviral vector (Cardarelli et al. 1995) by the calcium phosphate co-precipitation method and were grown in 60 ng/ml colchicine (Ueda et al. 1987).

Generation of expression vectors

Full-length cDNAs of rat Kir6.2 and SUR-1 were generously provided by Dr. Graeme I. Bell (Department of Medicine, University of Chicago). SUR-1 was originally cloned in pcDNA 3 under the cytomegalovirus (CMV) promoter along with the neomycin-resistance gene. In our laboratory, Kir6.2 was subcloned by ligating the 1.4-kb cDNA fragment flanking by EcoR I restriction sites, to an EcoR I digested pcDNA3.1/Zeo (+) expression vector, under the CMV promoter along with the Zeocin-resistance gene. The resulting expression vectors were confirmed by restriction endonuclease digestion and DNA sequence analysis. The constructs were also confirmed by expression in a coupled transcription-translation utilizing TNT rabbit reticulocyte lysates (Promega, Madison, Wis., USA) as instructed by the manufacturer. DNA sequencing was performed with Sequenase II kit. *Escherichia coli* strain DH5α was used for all plasmid transformations and propagations.

Transfection and selection of stable cell lines

PC12 cells were transfected with PCDNA3.1 vector or with Kir6.2 and SUR-1 vectors using lipofectamine reagent according to the manufacturer's directions. Briefly, 4×10^6 PC12 cells were seeded on 100-mm dishes (Falcon, Becton Dickinson, Franklin Lakes, N.J., USA). One day after seeding, the cells were washed in serum-free Opti-MEM I medium. Plasmid DNAs (5 μg each/800 μl) and lipofectamine (50 μl/800 μl) in Opti-MEM I medium were mixed and incubated for 45 min at room temperature before being added to the cells. Forty-eight hours after transfection, cells were replated at a splitting ratio of 1:10 into growth medium containing 0.5 mg/ml G418 and 0.25 mg/ml Zeocin for Kir6.2+SUR-1-transfected cells, 0.25 mg/ml Zeocin alone for PCDNA3.1- and Kir6.2-transfected cells and 0.5 mg/ml G418 for SUR-1-transfected cells. Transfected cells were cultured for 5 weeks with a change of medium every 3 days. Colonies were removed with cloning cylinders, grown in a selecting media and examined for Kir6.2 and SUR-1 mRNA expression.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted and isolated from PC12 cells using RNA ISOLATOR (Genosys Biotechnology, The Woodlands, Tex., USA). RNA (2 μg) was reverse transcribed to a single strand DNA (cDNA) using reverse primers and MoMLV-RT. Portions (20 ng) of the cDNA pool were amplified using Kir6.2, SUR-1, SUR-2, MRP1, MRP2 or β-actin primers based on reported sequences; Kir6.2, f-5′-CGAGGTCCAGGTGACCATTGG-3′ (at 417) and r-5′-TGCGGTCCTCATCAAGCTGGC-3′ (at 1082) (accession No. U44897); SUR-1 f-5′-TCGCGCTGTGCCTTGTCACC-5′ (at 3483) and r-5′-ATGAGTACCACGCATGCTCCG-3′ (at 4021) (accession no. L40624); SUR-2 f-5′-TGCGACATTTGTGACACATG-3′ (at 1870) and r-5′-CGTAAGCCACAGAATACCTGC-3′ (at 2369) (Chutkow et al. 1996). Rat β-actin f-5′-TTGTAACCAACTGGGACGATATGG-3′ (at 1552) and r-5′-GATCTTGATCTTCATGGTGCTAGG-3′ (at 2991) (Nudel et al. 1983). MRP1: f-5′-CTGCACCTAGACCTGCT-3′ (at 3337) and r-TCCAGGCGCTTCAGCT (at 3627), MRP2: f-5′-TGAGTGCTTGGACCAG-3′ (at 2987) and r-5′-CTTCTGACGTCATCCTCAC-3′ (at 3772) (Schaub et al. 1997). PCR was performed using a Perkin-Elmer Cetus thermal cycler in a volume of 100 μl containing cDNA, 10 μM deoxynucleoside triphosphates (dNTP), 100 mM KCl, 200 mM TRIS-HCl (pH 8.75), 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin (BSA), 10 pmol of each primer and 3 units of TaqPlus long PCR system. The PCR conditions were: for Kir6.2, SUR-1 and β-actin primers initial denaturation at 94 °C for 5 min followed by 35 (Kir6.2, SUR-1) or 27 (β-actin) cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 2 min and extension at 72 °C for 3 min, with a final extension at 72 °C for 10 min. For SUR-2 and MRP1/MRP2, initial denaturation was at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C (60 °C for MRPs) for 45 s and extension at 72 °C for 45 s (90 s MRPs), with a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis in 2% agarose gel, and visualized by ethidium bromide staining.

Distribution of metabolites between cells and culture medium

Cells ($1–2\times10^6$) were plated on poly-L-lysine-coated 35-mm tissue culture dishes 48–72 h prior to the release experiments. The serum-containing media was then removed and replaced with HEPES-

buffered saline having the following composition (mM): NaCl 115, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5.5, NaH_2PO_4 1, HEPES 15 buffer (pH 7.2). After 15-min adaptation, the HEPES-buffered saline (HBS) solution was removed and replaced with fresh HBS solution containing either no drug or the selected agent and the incubation at 37 °C continued for 15 min. When Cl^- was omitted, it was replaced with isethionate. At the end of 15 min, 150 µl of the medium was transferred into tubes containing 15 µl 0.1 M perchloric acid containing 1% ethanol and 0.02% EDTA for the measurement of the released catecholamines and metabolites. Cellular contents of these substances were determined by HPLC/ECD (see Biochemical studies) after cell lysis by 0.1 M $HClO_4$ containing 0.5% Na_2EDTA and 0.1% $Na_2S_3O_5$. PC12 cell retention of each of the acidic metabolites was expressed as the percentage of the total acidic metabolites in the culture dish found in the cells after 15 min incubation, i.e. 100×cell content/(cell+media contents). Differences in the percentage of the ADMs retained in the cells was taken as a measure of the effect of the treatment upon efflux of the metabolite from the cells into the medium.

In other experiments, the cells ($1–2\times10^6$) were incubated for 30 min in HBS containing 100 µM HVA or DOPAC. To examine the effects of glipizide (a "second generation" sulfonylurea) on HVA and DOPAC accumulation during exposure to the high ADMs concentrations (loading), the drug was added to the incubation medium along with HVA. The cells were washed 3 times with warm phosphate-buffered saline (PBS, pH 7.4) and harvested for determination of DOPAC and HVA. The effects of glipizide on the rates of efflux of the acids was assessed after the loading cells with HVA or DOPAC. The loaded cells were washed 3 times with warm PBS (pH 7.4) and the incubation continued for varying intervals in HBS at 37 °C with or without glipizide (100 µM). The residual accumulated ADMs in the cells at each time point were estimated by subtracting the amounts of the metabolites present when the cells had not been loaded with DOPAC or HVA. The percentage of the initial accumulated cellular contents of HVA or DOPAC was determined 2, 5, 10 and 15 min later to examine the effect of glipizide on the rate of efflux of the acids from the cells.

The effect of a high concentration of DOPAC on the accumulation of ^{3}H-glibenclamide by PC12 cells was examined by incubating PC 12 cells with a range of concentrations of DOPAC (10 µM–1 mM) for 30 min in media containing 10 µM ^{3}H-glibenclamide (50 Ci/mM), after which the cells were harvested (PHD Cell harvester, Cambridge Technology, Watertown, Mass., USA) by filtration (Glass Fiber Filter 240-1, Cambridge Technology). The cells on the filters were washed 3 times with cold PBS, liquid scintillation fluid was added (Cytoscint, ICN, Costa Mesa, Calif., USA) and the tritium retained by the cells was determined by liquid scintillation spectrometry.

Microdialysis Studies

Animals and surgical preparation

The Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke, National Institutes of Health approved the experimental procedures. Male Sprague-Dawley rats (250–300 g, Taconic Farms, Germantown, N.Y., USA) were housed two or three per cage with room temperature set at 20 °C, food and water available ad libitum, and a 12-h light/dark cycle for at least 5 days before the acute experiment, which was performed during the light period. Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and each animal was placed in a stereotaxic frame (David Kopf Instruments, Tujunga, Calif., USA), with the incisor bar 3.1 mm below the interaural line. The skull was exposed, and a small hole was drilled. The microdialysis probe (4 mm active surface; molecular weight cutoff 20 kD; CMA/Microdialysis, Acton, Mass., USA) was inserted at coordinates corresponding to the striatum (Paxinos and Watson 1982). Coordinates of the probe tip with respect to the bregma were: AP +0.2; L 2.9; V –8.4 mm. The implanted microdialysis probe was anchored to the skull with acrylic dental cement. The animals were allowed to recover overnight with water and food provided ad libitum.

Microdialysis procedure

Using a high-precision pump (CMA 100, CMA/Microdialysis), the microdialysis probe was perfused (2 µl/min) with artificial cerebrospinal fluid (CSF) which contained 122 mM NaCl, 3 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM KH_2PO_4, mM 25 $NaHCO_3$ (pH 7.4). The effluent microdialysates were collected at 20-min intervals into vials that contained 10 µl 0.1 M perchloric acid with 1% ethanol and 0.02% EDTA. After each 20-min collection period, the dialysate was frozen rapidly in dry ice and stored at –70 °C until assayed.

Experimental protocol

The morning after placement of the microdialysis probe, dialysis was initiated. After a 2-h equilibration period, baseline samples were obtained. Dialysate samples were collected in 20-min fractions during the entire interval (220 min) of the experiment (three baseline samples and eight samples after the beginning of drug administration) and frozen at –80 °C until analyzed for DOPAC and HVA as described below. Two experimental groups were studied. One group (n=3) received glipizide via the microdialysis probe (1 mM in artificial CSF, for 80 min); the second group (n=3) received probenecid via the microdialysis probe (1 mM in artificial CSF, for 80 min). At the end of each experiment, the dialysis probe was perfused with a solution of toluidine blue for 5 min. After the animal was killed by an injection (i.p.) of an overdose of pentobarbital, the brains were removed and placed into 10% paraformaldehyde solution in PBS for later histological verification of probe position.

Biochemical studies

Aliquots (10–100 µl) of media or cell lysates were assayed for catecholamines and metabolites by high-pressure liquid chromatography with electrochemical detection (HPLC/ECD). Dopamine and its metabolites were measured using an Ultrasphere C-18 ion-pair column, 5 µ, 4.6 mm×25 cm (Beckman, 235329): Waters 717 plus autosampler, Waters 510 pump at 0.8 ml/min, and an amperometric electrochemical detector (EiCom CB-100) with the analytical cell set at a potential of 0.78 V. The mobile phase contained 1.4 g/l L-heptanesulfonic acid sodium salt, 0.085 g/l EDTA, 1% triethylamine and 2.5% acetonitrile; and the pH was adjusted to 2.6 with 6.5 ml of 85% phosphoric acid. The detector output was recorded and analyzed using the Waters Millennium 2010 Chromatography Manager.

Fluorescent drug accumulation

Cells to be examined were removed from the culture flasks; washed and resuspended in Isscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% PBS. Subsequently, 500,000 cells were incubated in 5 ml IMDM (prewarmed to 37 °C) containing 5% FBS and rhodamine 123 (0.5 µg/ml), with or without the drug to be tested, for 30 min at 37 °C in 6-ml polystyrene tubes. The cells were resuspended in 4.5 ml substrate-free medium, with or without the drug and incubated for an additional 30 min at 37 °C. After centrifugation at 200 *g* for 5 min, the medium was removed by aspiration, and the cells resuspended in 350 µl ice-cold PBS and the rhodamine 123 content analyzed using a fluorescence-activated cell sorter.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

SDS-PAGE and immunoblot analyses were performed as described (Hrycyna et al. 1998). For P-glycoprotein and MRP1 analysis, samples were incubated in Laemmli SDS-PAGE sample-loading buffer (Laemmli 1970) at room temperature for 30 min prior to electrophoresis. The primary antibody, mAb C219 (Georges et al.

1990), was used at a 1:1500 dilution in 5% (w:v) nonfat dry milk dissolved in PBS containing 0.05% (v:v) Tween 20 (PBST). Secondary antibody solution was a 1:3000 dilution of goat anti-rabbit IgG conjugated with peroxidase in 5% (w:v) nonfat dry milk dissolved in PBST. MRP1 was detected using mAb rat anti-human R1 (Kamiya Biomedical, Thousand Oaks, Calif., USA) at a 1:3000 dilution and same concentration of secondary antibody.

Materials

NIH3T3 cells were a gift from Dr. Suresh Ambudkar, DBS, NCI, NIH. Full length cDNAs of rat Kir6.2 and SUR-1 were generously provided by Dr. Graeme. I. Bell (Department of Medicine, University of Chicago). DMEM, FBS, NGF, G418, Lipofectamine, Opti-MEM I medium and MoMLV-RT were purchased from Gibco (Gaithersburg, Md., USA). PcDNA3 and pcDNA3.1/Zeo (+) expression vectors were purchased from Invitrogen (Carlsbad, Calif., USA). Sequenase II kit was purchased from United States Biochemical (Cleveland, Ohio, USA). *Eschericia coli* strain DH5α and TaqPlus long PCR system were purchased from Stratagene (La Jolla, Calif., USA). TNT rabbit reticulocyte lysates was purchased from Promega. RNA ISOLATOR was purchased from Genosys Biotechnology. mAb rat anti-human R1 was purchased from Kamiya Biomedical (Seattle, Wash., USA). All other reagents were purchased either from Sigma (St. Louis Mo., USA) or from Fisher Scientific (Pittsburg, Pa., USA).

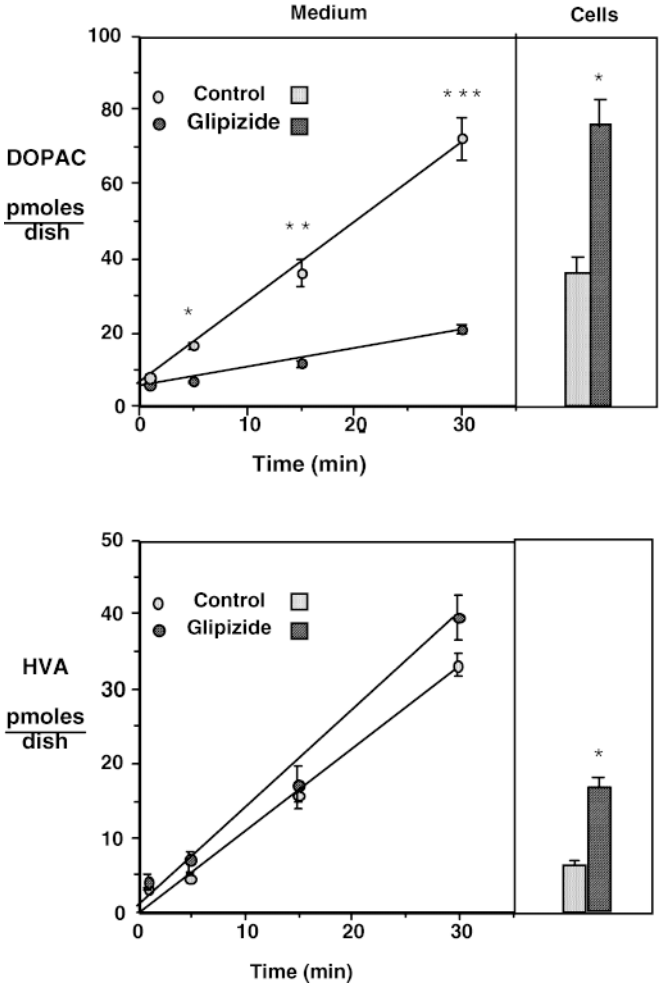


Fig. 1 Effect of glipizide (100 μM) on formation and distribution between cells and medium of 3,4-dihydroxyphenylacetic acid (*DOPAC*, *upper panel*) and homovanillic acid (*HVA*, *lower panel*). Cells (1–2×10^6) were plated and cultured for 48 h before being transferred to fresh media. Aliquots (150 μl) of the medium were collected at various intervals (5, 15, and 30 min) and analyzed for DOPAC and HVA. At the end of the experiment the cells were harvested and their HVA and DOPAC contents was determined. Results are mean values±SEM for groups of 5 or 6 wells. ***P<0.001, **P<0.01, *P<0.05 vs. absence of glipizide (Student's t-test)

Data analysis and statistics

The significance of the difference in ADM retention between control and treated groups was determined by analysis of variance and Dunnett's multiple comparisons test. Microdialysis data were expressed as percentage from basal level (average of three basal collection intervals). The significance of changes in microdialysate concentrations of DOPAC and HVA within the treated groups before and after drug administration was evaluated using ANOVA followed by the Tukey-Kramer multiple comparisons test. The effect of glipizide on DOPAC and HVA retention in HVA loading experiments was evaluated by ANOVA followed by Dunnett's test.

Results

Effect of glipizide on DOPAC and HVA retention in PC12 cells

Incubation of undifferentiated PC12 cells in HBS resulted in a time-dependent increase in levels of DOPAC and

Table 1 Treatment-induced increments in retention of 3,4-dihydroxyphenylacetic acid (*DOPAC*) and homovanillic acid (*HVA*) in PC12 cells. Results are expressed as changes in percentage of the acidic metabolite retained in the cells following 15 min incubation: (100×cell content/cell+media contents). Control levels were 26±1.1and 14±1.1 (SEM) % for DOPAC and HVA, respectively (*TTX* tetrodotoxin)

Treatment	*n*	DOPAC	HVA
Glibenclamide 100 μM	10	68 ±0.9**	18.8±0.9**
Glipizide 100 μM	30	49.2 ±1.1**	18.1±1.1**
Tolbutamide 500 μM	5	29.6 ±0.6**	9.3±1.2**
Tolbutamide 100 μM	5	14.8 ±0.8**	3.4±1.8
Diazoxide 100 μM	10	20.3 ±1.3**	12.2±2.7
Phentolamine 100 μM	4	0.8 ±0.3	–6.3±0.8*
Cromakalim 100 μM	4	1.4 ±0.5	–6.0±2.0
Forskolin 1 μM	5	–0.2 ±1.6	–3.5±0.6
Cl^- omitted	4	–1.2 ±0.5	5.7±0.7*
Cyclosporine 100 μM	5	0.64±2.9	3.9±2.1
Verapamil 100 μM	5	16.7 ±0.3**	2.3±0.6
Probenecid100 μM	4	23.8 ±0.9**	11.8±1.8**
Genistein 100 μM	4	26.8 ±1.7**	3.6±1.5
Indomethacin 100 μM	4	51.6 ±0.9**	13 ±0.4**
ouabain 50 μM	5	3.3 ±2.8	–0.5±2.1
TTX 6 μM	5	4.9 ±3.8	1.8±3.2
4 °C	5	62.3 ±5.1**	74.9±2.8**
rotenone 50 M	5	36.2 ±9.05 **	27.8±6.2**
KCl 56 mM)	5	–7.3 ±2.1**	–7.3±2.1**

**P<0.01, *P<0.05 vs. control (one-way ANOVA followed by Dunnet's test for multiple comparisons with a control group)

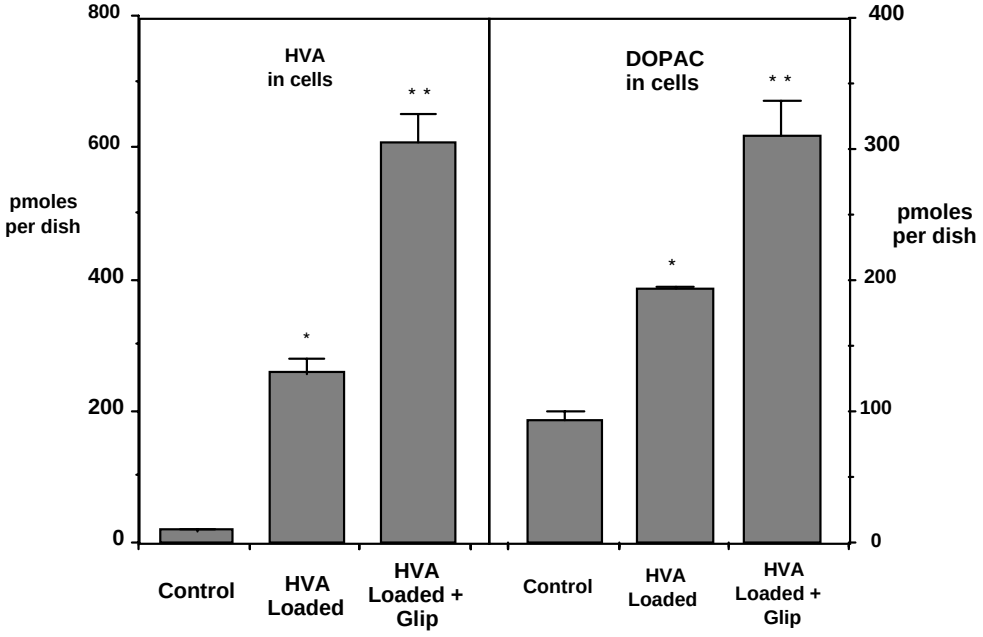


Fig. 2 Effect of HVA loading on HVA and DOPAC PC12 contents. Cells were incubated in medium containing 10^{-4} M HVA, with or without glipizide (*Glip*), for 30 min. At the end of the incubation period, cells in each of the dishes (n=4–5 per group) were washed as described in Methods and harvested for determination of their HVA and DOPAC contents. Means±SEM for groups of five or six wells. $^{**}P<0.001$, $^{*}P<0.05$ vs. control (ANOVA followed by Dunnet's test)

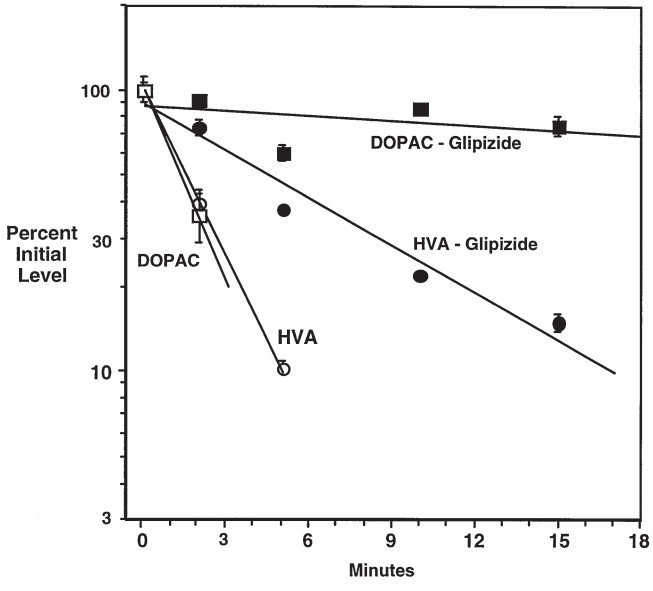


Fig. 3 Effect of glipizide (100 μM) on the retention of DOPAC and HVA in PC12 preloaded with either DOPAC or HVA (10^{-4} M) for 30 min. In one group of cells, media containing DOPAC also contained 100 μM glipizide. The HVA or DOPAC-containing media were replaced with fresh HVA-free medium containing 100 μM glipizide or no drug and the incubation was continued. DOPAC or HVA retention was measured at 0, 2, 5, 10, and 15 min following the end of incubation and corrected for the amount of the acid metabolite present in cells that had not been incubated in media containing the acid. Data for each group (n=4) are presented as percentages of initial levels; 161±8.8 pmol (without glipizide) and 306±35 (with glipizide) for HVA and 130±8.3 pmol (without glipizide) and 348±18.6 pmol (with glipizide) for DOPAC. Half-times for decline in cell metabolite contents in glipizide-containing and in drug-free media were calculated from the slopes obtained by linear regression analysis of the logarithm of the fraction of the initial values versus time

HVA in the medium (Fig. 1). In the presence of glipizide (100 μM), the rate of DOPAC accumulation in the medium was markedly reduced and that in the cells increased. The percentage of the total DOPAC retained in the cells was increased from 33.3 to 78.2% (an increase of 44.9%), indicating that glipizide decreased DOPAC efflux from the cells into the medium. Although more DOPAC was found in the cells, the total DOPAC recovered in the medium plus the cells was diminished. There was, however, a significant increase in total HVA formed (cells plus medium), from 39.6±1.5 to 55.8±3.9 pmol/dish. This increase in HVA was quantitatively similar to the decrease in the total DOPAC. Although in the presence of glipizide there was a slightly greater accumulation of HVA in the medium, the percentage of HVA retained in the cells was significantly increased. These results were confirmed in a series of 30 studies that included glipizide along with other agents (Table 1, see below). The mean increments in percentages of DOPAC and HVA retained in the cells in the series of 30 studies were 49.2±1.1 and 18.1±1.1, respectively (Table 1).

The effect of glipizide was concentration dependent; DOPAC accumulation was not apparent at 1 μM, but clearly evident above 10 μM and showed a maximum effect above 100 μM glipizide with an apparent IC_{50} of about 2×10^{-5} M (data not shown). The retention of DOPAC and HVA in the cells was similar in PC12 cells that were differentiated by incubation with NGF for 6 days (data not shown).

Since increased formation of HVA from DOPAC in the presence of glipizide partially obscured the effect of the drug on HVA transport out of the PC12 cells, to demonstrate more precisely the effect of glipizide on HVA efflux, PC12 cells were first loaded with HVA. Incubation of the cells in a medium containing a high concentration of HVA resulted in a striking increase (from 18.2±2.3 pmol to 250±22 pmol) in cellular contents of HVA; the

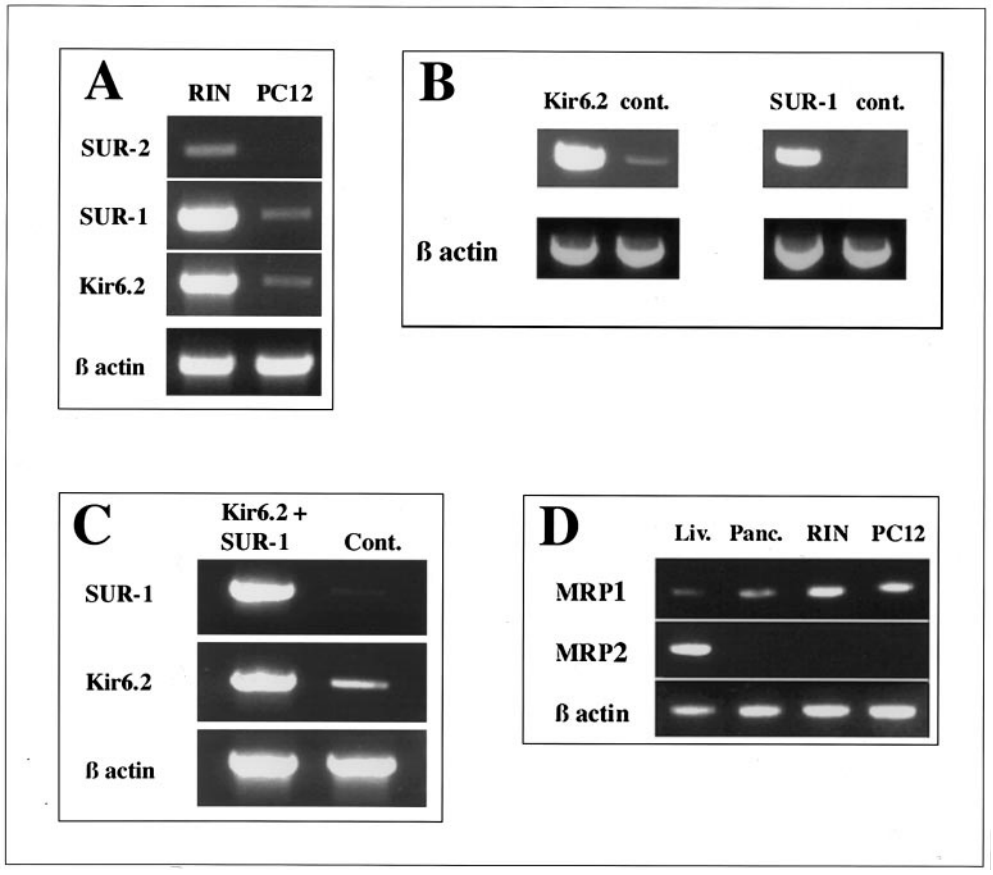


Fig. 4A–D Expression and over-expression of ATP-sensitive K^+ channel (K_{ATP}) subunits and multidrug resistance protein (*MRP*) -1 and -2 mRNA in PC12 cells. **A** mRNA expression of sulfonylurea receptor protein (*SUR-1*), Kir6.2 and SUR-2 in PC12 and RINm5F rat insulinoma (*RIN*) cells. **B** Over-expression of Kir6.2 mRNA in PC12 cells transfected with Kir6 vector (*left lane*) or SUR-1 (*right lane*). **C** Over-expression of both K_{ATP} channel subunits (Kir6.2+SUR-1). **D** Expression of MRP1 mRNA in PC12 cells. Shown is an agarose gel of amplification products from PCR of 20 ng of cDNA pool (*cont.* control cells, *PC12* cells transfected with vector alone, *Panc.* rat pancreas cDNA pool, *Liv.* rat liver cDNA pool)

cellular accumulation of HVA was enhanced (to 612±42 pmol) in the presence of glipizide (Fig. 2). Loading the PC12 cells with HVA also increased the DOPAC content of the cells.

As shown in Fig. 3, after removal of glipizide from the medium, the efflux of DOPAC and HVA in DOPAC and HVA pre-loaded PC12 cells was very rapid ($t_{1/2}$ of 0.6 and 1.5 min, respectively). Glipizide strikingly reduced the rate of efflux of DOPAC (to a $t_{1/2}$ of 55 min) and also diminished efflux rate of HVA by approximately 73% (to a $t_{1/2}$ of 5.5 min).

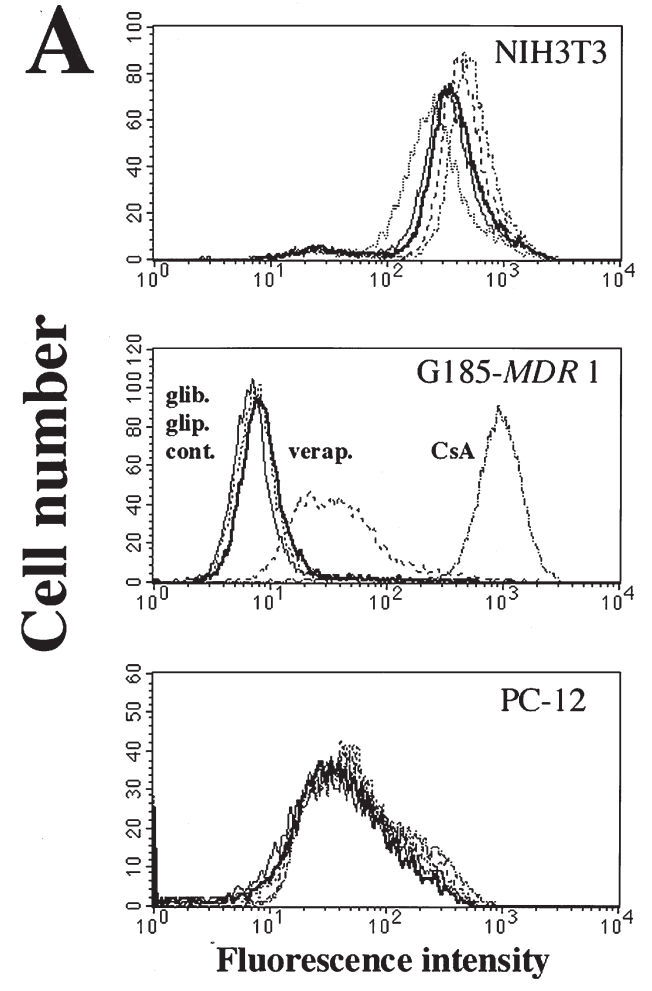
Effect of DOPAC on accumulation of ^{3}H-glibenclamide

Accumulation of ^{3}H-glibenclamide by PC12 cells was unaffected by incubation with concentrations of DOPAC ranging from 10 µM to 1 mM (data not shown), indicating that DOPAC interfered with neither glibenclamide entry into nor exit from PC12 cells.

Effects of drugs that affect ABC transporters on DOPAC and HVA retention in PC12 cells

The effects of a variety of sulfonylureas and other agents on retention of ADMs in PC12 cells were examined to pharmacologically characterize the transporter (Table 1). Glipizide and glibenclamide were more potent than tolbutamide (glibenclamide>glipizide>>tolbutamide) in blocking the efflux of ADMs. The magnitudes of effects of the drugs on DOPAC retention were much greater than on HVA retention. Diazoxide (K_{ATP} channel opener) appeared to be somewhat more effective than tolbutamide (K_{ATP} channel blocker) in diminishing the efflux of ADMs. However, phentolamine, which blocks the K_{ATP} channel by interacting with the Kir6.2 motif (Proks and Ashcroft 1997), and cromakalim, a K_{ATP} channel opener (Quast and Cook 1989), had no significant effects on the efflux of ADMs.

The cystic fibrosis transmembrane receptor (CFTR) is a member of the ATP-binding cassette family of transporter proteins (Higgins 1995). Because CFTR-modulated ATP release is dependent on both extracellular [Cl^-] and cAMP activation (Jiang et al. 1998), we examined the effects of omission of Cl^- and forskolin on accumulation of DOPAC. Neither omission of Cl^- from the medium nor stimulation of cAMP synthesis by forskolin significantly altered ADM levels or glipizide-induced increments in cell ADM levels (data not shown). Cyclosporine A, a potent MDR blocker (Foxwell et al. 1989), did not affect ADM efflux from PC12 cells, whereas verapamil was nearly as effective as diazoxide in diminishing ADM efflux. Probenecid, an acid metabolite carrier blocker (Gordon et al. 1976) and other MRP blockers, e.g., genistein (Versantvoort et al. 1994) and indomethacin (Hollo et al. 1996) enhanced ADM retention in the cells (Table 1).



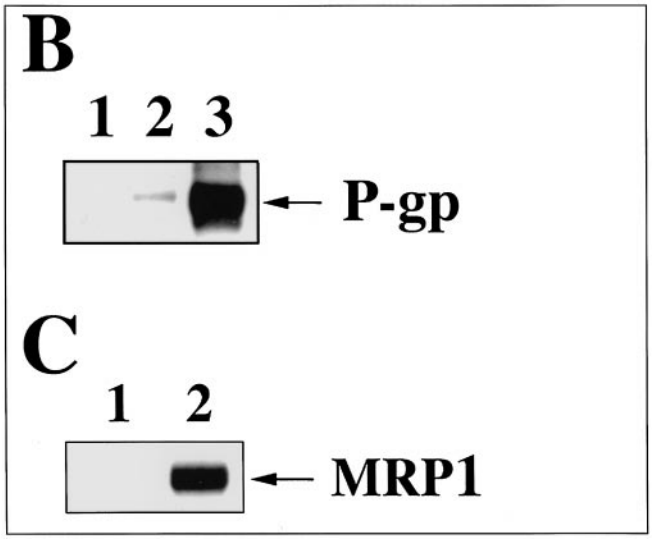


Fig. 5 **A** Effects of drugs on fluorescent rhodamine accumulation in the *MDR*1-expressing cell line G185-*MDR*1, in NIH3T3 and in PC12 cells following incubation with vehicle, 100 μM glibenclamide (*glib.*), 100 μM glipizide (*glip.*), 20 μM verapamil (*verap.*) and 10 μM cyclosporine A (*CsA*). The number of cells (*ordinate*) expressing a given fluorescence intensity (*absissa*) is illustrated. **B** Immunoblot analysis of P-glycoprotein (*P-gp*) protein expression in: *1* PC12 cells, *2* NIH3T3 cells, *3* NIH3T3-G185-*MDR*1 cells: 40 μg total protein was loaded on 8% SDS-PAGE gels and subjected to immunoblot analysis using monoclonal antibody C219 (1:1500). **C** Expression of MRP1 in NIH3T3 and NIH3T3-MRP1 cells: 40 μg total protein was loaded on 8% SDS-PAGE gels and subjected to immunoblot analysis using monoclonal antibody mAb R1 (1:3000)

Over-expression of Kir6.2, SUR-1 and MRP1 does not affect transport of ADMs

Compared with RINm5F (rat insulinoma cell line) cells, PC12 cells expressed relatively small amounts of mRNAs for both subunits of the pancreatic K_{ATP} channel, Kir6.2 and SUR-1 and undetectable amounts of SUR-2 (Fig. 4A). Using electrophysiological methods, we could not demonstrate active K_{ATP} channels in these cells (I. Lamensdorf, C. Hrycyna, L-P. He, A. Nechushtan, O. Tjurmina, J. Harvey-White, G. Eisenhofer, E. Rojas, I. Kopin, unpublished observation). When we over-expressed Kir6.2, SUR-1 (Fig. 4B) or both subunits of the K_{ATP} channel in PC12 cells (Fig. 4B), neither basal nor glipizide-induced increments in intracellular ADM levels were significantly different than in PC12 cells stably expressing the vector alone (data not shown). MRP1 was found to be expressed in PC12 cells as well as in RINm5F cells, pancreas and liver (Fig. 4D), but MRP2 could be detected only in the liver. There was no significant difference in DOPAC loading into MRP1-transfected and NIH3T3 cells and glipizide had no significant effect on DOPAC efflux in either of these cell lines (data not shown).

The transporter for ADMs is not P-glycoprotein

Immunoblot analysis revealed that PC12 cells do not express detectable amounts of P-glycoprotein (Fig. 5B). NIH3T3 cells, which lack the MRP transporter, accumulated rhodamine; but the amount of rhodamine that accumulated was not influenced by exposure to verapamil, cyclosporine A, glipizide or glibenclamide (Fig. 5A). Cyclosporine A and verapamil, but not glipizide or glibenclamide, blocked rhodamine exclusion from NIH3T3 cells expressing wild type human P-glycoprotein, (NIH3T3-G185-*MDR*1) (Fig. 5A). The accumulation of rhodamine 123 in PC12 cells was not affected by any of the four drugs tested (Fig. 5A).

Transport of ADMs is an energy dependent process

PC12 cells express both a tetrodotoxin (TTX)-sensitive Na channel (Rudy et al. 1987) and a ouabain-sensitive

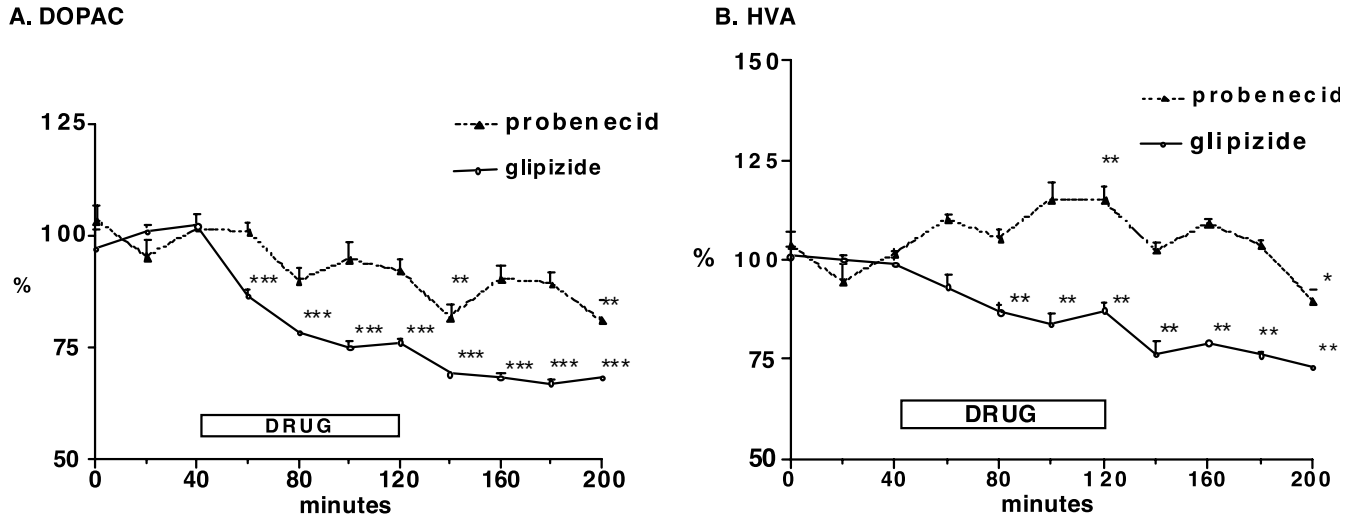


Fig. 6 Microdialysate levels of DOPAC and HVA during perfusion of rat striatum with l mM probenecid (n=3) or l mM glipizide (n=3). Microdialysis probes were inserted into the striatum of rats as described in Methods. After collection of control samples, perfusion was continued with artificial cerebrospinal fluid containing one of the drugs. Microdialysate HVA and DOPAC levels were determined and expressed as a percentage of the mean of three baseline values. The significance of probenecid and glipizide effects on ADM efflux was determined by ANOVA followed by the Tukey-Kramer multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001 vs. baseline

Na^+/K^+-ATPase (Mackler et al. 1998). Modulation of Na^+/K^+-ATPase by ouabain, blockade of the Na^+ current with TTX or depolarizing the cells with high concentrations of KCl did not elevate ADM retained in the PC12 cells (Table 1). However, depleting the ATP levels by addition of rotenone and omission of glucose from the media (de Weille et al. 1989) or incubating the cells at 4 °C significantly attenuated ADM efflux, consistent with an energy-dependent transport process (Table 1).

Glipizide significantly decreases basal extracellular DOPAC (F=46, P<0.0001) and HVA (F=25.4, P<0.01) levels in vivo. Glipizide (1 mM) reduced microdialysate levels of DOPAC more effectively than did probenecid, for which the response was significant only at 140 min (F=5.9, P<0.0001; P<0.002) (Fig. 6A) and also reduced microdialysate HVA levels, whereas probenecid slightly increased the extracellular HVA levels, which were significantly greater (F=5.1, P<0.001) at100–120 min from the beginning of the experiment (P<0.01) (Fig. 6B).

Discussion

This study demonstrates that ADMs are secreted from PC12 cells in vitro by a sulfonylurea-sensitive, energy-dependent transporter. The pharmacological properties of the ADM transporter suggest that it may belong to the ABC transporter family, but that it differs from known ABC anion transporters and from the SUR component of K_{ATP} channels. Evidence for the presence of a similar sulfonylurea-sensitive transporter in rat brain was obtained using microdialysis.

The glipizide-induced reduction in the rate of DOPAC efflux out of the PC12 cells into the medium, which was accompanied by an increase in DOPAC retention in the cells, indicated the presence of a glipizide-sensitive transport system for DOPAC. In the presence of glipizide, there was a net decrease in total DOPAC recovered from the cells and medium. The decrease in total DOPAC was attended by a corresponding increase in total HVA, indicating that a portion of the retained DOPAC was O-methylated. Since *O*-methylated catechols are more lipophilic than the corresponding catechols (Henseling et al. 1978), exit of HVA is not as dependent on the transport system as is DOPAC. The increased formation of HVA in the cells resulted in a slight increase of HVA in the medium. However, reduced active export of HVA resulted in a greater proportional increase in content of HVA in the cells than in the medium. Since a portion of the DOPAC retained in the cells is *O*-methylated to HVA, the degree of inhibition of DOPAC efflux was probably underestimated.

During loading, HVA readily entered the cells and glipizide, rather than diminishing entry of HVA into the cells, enhanced the accumulation of the exogenous HVA. If HVA transport were bi-directional, glipizide would be expected to prevent accumulation of HVA, thus these results indicate that HVA transport was unidirectional.

When exogenous HVA accumulated in the cells, DOPAC levels also increased, even in the absence of glipizide, presumably as a result of competitive inhibition by HVA of DOPAC efflux. Although *O*-methylated catechols can be demethylated (Kopin et al. 1961), this is a slow, minor pathway and the resulting catechol is rapidly re-*O*-methylated. Thus, the increment in DOPAC cannot be attributed to *O*-demethylation of HVA.

After loading with exogenous HVA or DOPAC, efflux of the acids from PC12 cells is extremely rapid, presumably a result of active export of the acidic metabolites. Glipizide had a much greater effect in diminishing efflux of DOPAC from the cells than of HVA as expected from

their relative dependency upon transport for exit from the cells (see above).

Because of the well-known effects of sulfonylureas on K_{ATP} channels through their interaction with the SUR subunit, we first considered the possibility that K_{ATP} channels or SUR was responsible for ADM transport. As indicated earlier, SUR appears to be a member of the ABC transporter family (see Tusnady et al. 1997). It is unlikely, however, that K_{ATP} channels or their one of their SUR subunits mediates the effects of sulfonylureas on ADM efflux since the concentration of sulfonylureas required to inhibit the K/ATP channels are in the nanomolar range for glibenclamide and glipizide, whereas the effects of these drugs on ADM efflux required as much as 10 μM. Furthermore, consistent with the reports by Latha et al. (1994) and de Weille et al. (1989), we could not demonstrate any K_{ATP} channels in PC12 cells. Also, after glipizide incubation, there were no significant differences in DOPAC and HVA efflux from transfected PC12 cells made to express K_{ATP}s or one of its subunits.

The relative potencies (rank order) of the sulfonylureas in blocking ADM efflux appears to parallel their potency for inhibition of the pancreatic β-cell K_{ATP} channels, albeit requiring about 1000-fold higher concentrations to inhibit transport than to block K_{ATP} channels. This similarity of relative potencies suggests that the molecular structure of the transporter might include a structural sulfonylurea-binding motif similar to that on SUR-1, the neuronal/pancreatic sulfonylurea receptor. Furthermore, the K_{ATP} channel opener, diazoxide, which also interacts with SUR-1, had similar effects on ADM outflow, whereas neither cromakalim, a K_{ATP} channel opener (Quast and Cook 1989) which interacts with SUR-2 (Hambrock et al. 1998; Schwanstecher et al. 1998), nor phentolamine, which blocks K_{ATP} channels directly by its interaction with the pore forming protein-Kir6.2 subunit (Proks and Ashcroft 1997) had any significant effect on DOPAC or HVA efflux.

In addition to their actions at K_{ATP} channels, sufonylureas act on some anion transporters. Sulfonylurea drugs have been shown to inhibit the ABC1 transporter as well as P-glycoprotein and the CFTR, each of which transport anions (Becq et al. 1997; Hamon et al. 1997; Golstein et al. 1999). Golstein et al. (1999) concluded that glibenclamide and a related compound appear to be general inhibitors of ABC transporters.

Although a direct interaction of the ADM transporter with ATP has not been demonstrated, the properties of the transporter are consistent with the properties of the ABC transporter family. The transport is unidirectional and energy dependent as indicated by attenuated ADM outflow when the temperature is lowered to 4 °C or when metabolism is disturbed by omission of glucose in the presence of the mitochondrial complex-1 inhibitor rotenone (Table 1). Furthermore, several drugs that block other ABC transporters, such as genistein, probenecid and indomethacin, which block MRPs, and verapamil, which blocks P-glycoprotein, a MRP, were found to block ADM efflux.

Although PC12 cells express MRP1 mRNA, it is unlikely that MRP1 is the ADM transporter since DOPAC efflux from NIH3T3 expressing MRP1, a cell line known to transport anions (Cardarelli et al. 1995) was not affected by glipizide. PC12 cells do not express detectable amounts of P-glycoprotein, so this transporter can not be responsible for ADM efflux. Furthermore, sulfonylurea drugs did not block rhodamine exclusion from a MRP-positive cell line, and cyclosporine A, which efficiently blocks the MDR transporter, did not alter ADM efflux from PC12 cells. Both CFTR and the ABC1 protein are blocked by the sulfonylureas (Sheppard and Welsh 1992; Becq 1997). CFTR-modulated ATP release is dependent on both extracellular [Cl^-] and cAMP activation (Jiang et al. 1998), but neither omission of Cl^- nor forskolin affected the transport of DOPAC. It is also unlikely that the ABC1 protein is responsible for the transport since elevation of cAMP with forskolin did not alter ADM efflux (basal or in response to glipizide).

Transport of ADMs appears to be independent of Na^+ and K^+ gradients since ouabain, TTX, or depolarizing concentrations of potassium had no significant effects on the transport of the acids. The failure of depolarizing concentrations of KCl to elevate ADM retained in the PC12 cells indicates that this transporter differs from that postulated by Miyamoto et al. (1993) on the basis of effects of high potassium concentrations on microdialysate concentrations of DOPAC and HVA.

The results of microdialysis studies show that a sulfonylurea-sensitive ADM transporter is also active in the brain in vivo. Local administration of glipizide or probenecid via a microdialysis probe significantly reduced striatal microdialysate DOPAC levels in freely moving rats. However, HVA levels were reduced only in glipizide treated rats. This suggests that glipizide is a specific blocker of the neuronal ADM transporter, unlike probenecid which blocks organic acid transport by a variety of transporters. In vivo, the effects of probenecid on the ADM transporter in brain cells are apparently masked by its blockade of the organic anion transporter located at the blood-brain barrier (Gordon et al. 1976). Enhanced *O*-methylation of retained intracellular DOPAC to HVA may also contribute to maintenance of extracellular HVA levels after administration of probenecid. The presence of an ATP-dependent transporter that is responsible for export of ADMs from brain cells, including dopaminergic neurons, may explain the ischemia-induced increase in tissue and decrease in extracellular levels of acid dopamine metabolites in the rat striatum demonstrated by Phebus et al. (1995).

An energy-dependent transporter that rapidly extrudes DOPAC may be important to limit the concentrations of DOPAC in the cytoplasm of dopaminergic cells. DOPAC (and other catechols) can directly modulate tyrosine hydroxylase (TH) activity (Laschinski et al. 1986). Also, DOPAC can promote free radical formation and cytotoxicity (Shiekhattar et al. 1992) and outside of the dopaminergic neurons, DOPAC can act as an antioxidant to protect against free radical damage (Liu and Mori 1993).

In summary, we have demonstrated the presence of an apparently novel transporter that exports DOPAC and

HVA from PC12 cells into the medium in vitro. Based on pharmacological studies, we suggest that this transporter may be a new member of the ABC transporter superfamily. The effects of a sulfonylurea on microdialysate levels of DOPAC suggested that this transport process is also present in rat striatum.

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